PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

JOHN & KERNICK
An Intellectual Property Office of
Bowman Gilfillan Inc.
P.O. Box 3511
1685 Halfway House
AFRIQUE DU SUD

Date of mailing (day/month/year)
11 April 2002 (11.04.02)

Applicant's or agent's file reference
P14078PC00

IMPORTANT NOTIFICATION

International application No.
PCT/ZA00/00173

International filing date (day/month/year)
18 September 2000 (18.09.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

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NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

JOHN & KERNICK
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Bowman Gilfillan Inc.
P.O. Box 3511
1685 Halfway House
AFRIQUE DU SUD

Date of mailing (day/month/year)	
11 April 2002 (11.04.02)	
Applicant's or agent's file reference P14078PC00	**IMPORTANT NOTIFICATION**
International application No. PCT/ZA00/00173	**International filing date** (day/month/year) 18 September 2000 (18.09.00)

1. The following indications appeared on record concerning:

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PATENT COOPERATION TREATY

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NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

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Date of mailing (day/month/year)
03 août 2001 (03.08.01)

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P14078PC00

IMPORTANT NOTIFICATION

International application No.
PCT/ZA00/00173

International filing date (day/month/year)
18 septembre 2000 (18.09.00)

1. The following indications appeared on record concerning:

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PCT/ZA00/00173

PATENT COOPERATION TREATY

10/088627

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(PCT Administrative Instructions, Section 411)

To:

JOHN & KERNICK
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P.O. Box 3511
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Date of mailing (day/month/year) 29 August 2002 (29.08.02)	
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International application No. PCT/ZA00/00173	International filing date (day/month/year) 18 September 2000 (18.09.00)
International publication date (day/month/year) 22 March 2001 (22.03.01)	Priority date (day/month/year) 17 September 1999 (17.09.99)
Applicant BURTON, Stephanie, Gail et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

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3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

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Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
17 Sept 1999 (17.09.99)	99/5981	ZA	25 June 2002 (25.06.02)

The International Bureau of WIPO
34, chemin des Colombettes
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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

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NOTIFICATION OF ELECTION

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To:

Commissioner
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2011 South Clark Place Room
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in its capacity as elected Office

Date of mailing (day/month/year) 29 June 2001 (29.06.01)	
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International filing date (day/month/year) 18 September 2000 (18.09.00)	**Priority date** (day/month/year) 17 September 1999 (17.09.99)
Applicant BURTON, Stephanie, Gail et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

12 April 2001 (12.04.01)

[ ] in a notice effecting later election filed with the International Bureau on:

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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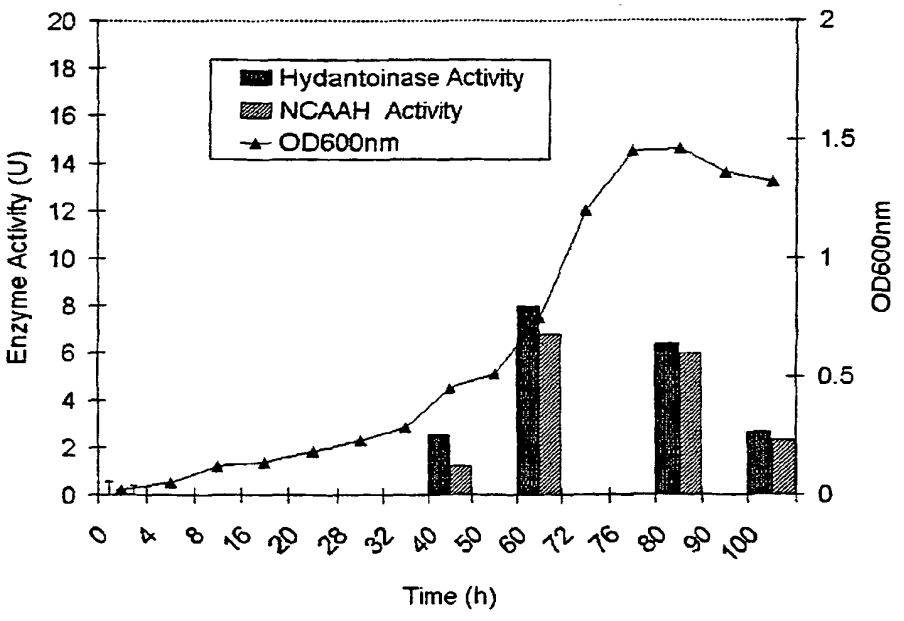
(54) Title: NOVEL MICRO-ORGANISMS, THEIR USE AND METHOD FOR PRODUCING D-AMINO ACIDS



(57) Abstract: The invention relates to novel micro-organisms which are simple to cultivate and their use in the production of D-amino acids, particularly micro-organisms suitable for the production of D-amino acids from corresponding hyantoins of *N*-carbamoylamino acids.

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NOVEL MICRO-ORGANISMS. THEIR USE AND METHOD FOR PRODUCING D-AMINO ACIDS

FIELD OF THE INVENTION

The invention relates to novel micro-organisms and their use in the production of D-amino acids. In particular, micro-organisms suitable for the production of D-amino acids from corresponding hydantoins or *N*-carbamoylamino acids. These novel micro-organisms are simple to cultivate and make possible high D-amino acids yields from different substrates.

BACKGROUND OF THE INVENTION

The importance of optically pure amino acids is primarily due to the use of D-amino acids, e.g. D-*p*-hydroxyphenylglycine, as side chains in semi-synthetic penicillins and cephalosporins (Syldatk *et al*, 1990). Optically pure amino acids also have applications in the production of other pharmaceuticals and flavourants (e.g. D-alanine in sweetners), pesticides (D-valine in the synthesis of insecticide fluvanilate), and as additives in animal feedstock (Polastro, 1989). Conventionally, D, L-5-substituted hydantoins have been used as starting materials for the chemical synthesis of D-amino acids. This process is cumbersome and inefficient since chemical synthesis results in an equimolar mixture of D- and L-amino acids requiring racemate resolution to obtain optically pure D-amino acids (Syldatk *et al.*, 1990). An alternative to chemical synthesis is the use of enzymatic conversion of hydantoins to their respective amino acids (Olivieri *et al.*, 1979). Biocatalytic conversions have major advantages: the enzyme systems are stereoselective and mild reaction conditions

result in a cheap industrial process with environmentally benign by-products and effluents (Santaniello *et al.*, 1992). The biocatalytic conversion of D,L-*p*-hydroxyphenylhydantoin to D-*p*-hydroxyphenylglycine has been listed as one of the main biocatalytic processes in the world market (Polastro, 1989).

The biocatalytic conversion of hydantoins to their corresponding amino acids is catalysed by two enzymes: first, an hydantoinase catalyses the ring-opening hydrolysis of the 5-substituted hydantoin to produce an *N*-carbamylamino acid in a reversible reaction. Classified as cyclic amidases (E.C.3.5.2), hydantoinases may be D-, L- or non-stereoselective. In the second reaction, the *N*-carbamylamino acid is converted to its corresponding amino acid either chemically, or through the action of a second enzyme, an *N*-carbamylamino acid amidohydrolase (E.C.3.5.1.6), which is usually stereoselective. (Olivieri *et al.*, 1979). While racemization of the hydantoins occurs spontaneously at alkaline pH, certain microbial systems include a D-racemase which converts L-5-substituted hydantoins to the corresponding D-enantiomers (Runser *et al.*, 1990; Hartley *et al.*, 1998).

D-selective hydantoin-hydrolysing enzyme systems have been identified in a variety of bacteria, including a *Pseudomonas* isolate (Ikenaka *et al.*, 1998), *Bacillus stearothermophilus* (Lee *et al.*, 1996), *Bacillus circulans* (Lukša *et al.*, 1997) and several *Agrobacterium* strains (Olivieri *et al.*, 1981; Runser *et al.*, 1990; Hartley *et al.*, 1998; Nanba *et al.*, 1998). The genes encoding one hydantoinase and three *N*-carbamylamino acid amidohydrolase enzymes from the Agrobacterium strains have been cloned and over-expressed in *Escherichia coli* (Durham and Weber, 1995; Buson *et al.* 1996; Grifantini *et al.*, 1998; Nanba *et al.*, 1998). DNA sequence analysis has revealed a high degree of amino acid homology between *N*-carbamylamino acid amidohydrolases from the Agrobacteria (Nanba *et al.*, 1998).

Characterisation of the enzyme system of A. *tumefaciens* RU-OR showed that enzymes activity was induced at high levels only when cells were grown in the presence of 2-thiouracil or hydantoin. Furthermore, maximum enzyme activity in cells grown in complete medium was detected in early stationary phase. (Hartley *et al.*, 1988). Similar observations have been made for hydantoin-hydrolysing enzyme systems from *A. radiobacter* (Deepa *et al.*, 1993), *Agrobacterium* sp. IP I-671 (Meyer

& Runser, 1993) and those of other bacteria with L-selective enzyme systems, such as *Arthrobacter crystallopoietes* (Möller *et al.*, 1988) An *A. tumefaciens* mutant, with inducer-independent production of hydantoinase and NCAAH, has been isolated by Hartley *et al.* (1998) and a similar mutant strain, *Arthrobacter* sp. DSM 9771, has been isolated by Wagner *et al.* (1996).

In this invention the word "constitutive" is to be understood to mean unregulated expression of enzymes; the word "expression" is understood to mean the production of a protein from a DNA template via transcription and translation; the word "activity" is understood to mean the ability of the hydantoinase and *N*-carbamylamino acid aminohydrolase enzymes to hydrolyse hydantoins to *N*-carbamylamino acids and amino acids and vice versa, respectively; the phrase "over-express" to mean levels of enzyme production in excess of those under the same conditions in the original isolate, and the phrase "enzyme system" is to be understood to include hydantoinase, *N*-carbamylamino acid amidohydrolase and hydantoin racemase enzymes which are capable of converting D- or L- or D,L-5-monosubstituted hydantoins or D- or L- or D,L- *N*-carbamoylamino acids to their corresponding, optically pure D-amino acids.

Recombinant systems for the over-expression of both hydantoinase and NCAAH enzymes in *E. coli* are known. However, reports of the production of insoluble aggregates and plasmid instability in cells over-expressing the NCAAH indicate that heterologous expression of these enzymes in *E. coli* may not be the ideal system. This has led to renewed interest in the use of homologous hosts for hydantoinase and NCAAH production, where the main problem is that enzyme activity needs to be induced and is confined to stationary growth phase under optimum growth conditions. This means that the levels of enzyme production per unit biomass in commercial strains remain relatively low. The re-introduction of a recombinant NCAAH gene under control of a constitutive promoter into *Agrobacterium* 80/44-2A resulted in high levels of biocatalytic activity.

The problems relating to genetically modified organisms and the obvious economic advantages of industrial strains that are not genetically modified, have led to the examination of the potential of mutant bacterial strains in the high-level production of hydantoinase and NCAAH enzymes.

OBJECT OF THE INVENTION

An object of the invention is the isolation of micro-organisms able constitutively to produce enzymes which convert racemic mixtures of 5-substituted hydantoins or N-carbamyl amino acids to D-amino acids and thereby, at least partially, to alleviate the problems associated with chemical synthesis of D-amino acids.

SUMMARY OF THE INVENTION

In accordance with the invention there is provided a biologically pure culture of a mutant strain of *Agrobacterium* RU-OR which constitutively expresses a stereoselective enzyme system which may be used in the enzymatic synthesis of D-amino acids.

Further in accordance with the invention there is provided a biologically pure culture of a glutamine synthesis-deficient micro-organism able constitutively to produce enzymes which convert racemic mixtures of 5-substituted hydantoins to D-amino acids.

Furthermore in accordance with the invention there are provided micro-organisms which are able to constitutively produce enzymes which convert racemic mixtures of *N*-carbamylamino acids to D-amino acids.

Further in accordance with the invention there is provided an isolated and purified enzyme system able to convert racemic mixtures of 5-substituted hydantoins to D-amino acids.

Still further in accordance with the invention there is provided an isolated and purified enzyme system able to convert racemic mixtures of *N*-carbamylamino acids to D-amino acids.

Furthermore in accordance with the invention there is provided a micro-organism for use in the production of D-amino acids for the production of pharmaceuticals,

alternatively agrochemicals, further alternatively for use in the production of D-amino acids for the production of pesticides, and still further alternatively for use in the production of D-amino acids for the production of feedstock additives.

The invention also extends to a growth medium to achieve over-expressed levels of hydantoinase and/or NCAAH enzyme activity during optimum culture conditions.

The invention also provides for a *N*-carbamylamino acid produced in accordance with the invention.

The invention also provides for a D-amino acid produced in accordance with the invention.

BRIEF DESCRIPTION OF THE FIGURES

In the accompanying Figures:

Figure 1 shows the DNA sequence of the 16S rRNA gene of *Agrobacterium* RU-OR;

Figure 2 shows hydantoinase and *N*-carbamylamino acid amidohydrolase activity in *Agrobacterium* RU-OR cells during mid-logarithmic phase during growth in HMM;

Figure 3 shows the effect of carbon and nitrogen source on hydantoinase and *N*-carbamylamino acid amidohydrolase activities in RU-OR cells;

Figure 4 shows that ammonia shock represses enzyme activity in wild-type *Agrobacterium* RU-OR cells;

Figure 5 shows that RU-ORPN1 cells constitutively express hydantoinase enzyme, but that the hydantoinase enzyme is inactive due to repression by ammonium in the growth medium;

Figure 6 shows that RU-ORPN1 cells constitutively express active *N*-carbamylamino acid amidohydrolase enzyme, while the wild type enzyme is repressed;

Figure 7 shows that hydantoinase activity in RU-ORPN1F9 cells is not sensitive to ammonia shock;

Figure 8 shows the levels of hydantoinase activity in RU-ORPN1F9 cells during mid-logarithmic growth phase compared with the levels in the wild-type RU-OR and mutant RU-ORPN1, when cells are grown under optimal growth conditions;

Figure 9 shows the levels of *N*-carbamylamino acid amidohydrolase activity in both RU-ORPN1 and RU-ORPN1F9 cells during mid-logarithmic growth phase compared with the levels in the wild-type RU-OR, when cells are grown under optimal growth conditions, and

Figure 10 shows the increase in specific hydantoinase activity per unit biomass in RU-ORPN1F9 cells in mid-logarithmic growth phase, with D,L-*p*-hydroxyphenylhydantoin as substrate, as compared with the specific hydantoinase activity in the wild-type RU-OR cells and RU-ORPN1 cells achieved during stationary phase.

DESCRIPTION OF ONE EMBODIMENT OF THE INVENTION

Several *Agrobacterium* strains have been reported to have hydantoin-hydrolysing activity. Among these are *Agrobacterium tumefaciens* 47 C, *Agrobacterium radiobacter* B11291 and *Agiobacterium* sp. IP I-671. *Agrobacterium radiobacter* B11291 and *Agrobacterium* sp IP I-671 also have *N*-carbamylamino acid and amidohydrolase activity. In the present invention, a novel *Agrobacterium* species (RU-OR) was isolated which is capable of producing a number of enzymes in amounts such that the cell mass has a high activity for the methods described herein.

CULTURE AND BIOCATALYTIC ASSAY CONDITIONS

Agrobacterium RU-OR and RU-ORPN1 cells grown to saturation in hydantoin minimal medium (HMM) broth. are diluted to OD_{600nm} = 0.02 in standard minimal

medium (MM) (MM per litre: 10g glucose; 0.011g $CaCl_2$; 0.02g $MgCl_2$; 60g Na_2HPO_4, 30g KH_2PO_4, 5g NaCl, 0.04g boric acid, 0.04g $MnSO_4$, 0.02g $(NH_4)_6Mo_2O_{24}.4H_2O$, 0.01g KI, 0.004g $CuSO_4$) supplemented with 1% hydantoin (HMM), 0.01% casamino acids (SMM), or $(NH_4)_2SO_4$. (AMM). Strain RU-ORPN1F9 cells are grown in HMM or SMM or AMM supplemented with 0.002% glutamine. Enzyme activity in *Agrobacterium* RU-OR cells was induced by growth in medium containing 0.1% thiouracil. Cells are harvested at OD_{600nm} = 0.5 – 0.8, pelleted by centrifugation, washed in 0.1 M PO_4 buffer pH 8.0 and resuspended in hydantoin or *N*-carbamylglycine reaction buffer at a final hydrated biomass concentration of 20 mg/ml (reaction buffer: either 50 mM hydantoin or 25 mM *N*-carbamylglycine in 0.1 M PO_4 buffer pH 8.0). Hydantoinase activity is measured as the sum of the concentration of *N*-carbamylglycine (µmol/ml) and glycine (µmol/ml) produced from 50 µmol/ml hydantoin in a 5 ml reaction volume after 6 h, shaking, at 40°C. *N*-carbamylamino acid amidohydrolase activity is measured as the concentration of glycine (µmol/ml) produced from 25 µmol/ml *N*-carbamylglycine in a 5 ml reaction volume after 6 h, shaking, at 40°C.

ISOLATION OF *AGROBACTERIUM* RU-OR, RU-ORPN1 and RU-ORPN1F9

Soil samples from the Eastern Cape environment were inoculated into hydantoin minimal medium (HMM) broth (per litre: 10g glucose; 0.011g $CaCl_2$; 0.02g $MgCl_2$; 60g Na_2HPO_4, 30g KH_2PO_4, 5g NaCl, 0.04g boric acid, 0.04g $MnSO_4$ 0.02g $(NH_4)_6Mo_2O_{24}.4H_2O$, 0.01g KI, 0.004g $CuSO_4$, 1% hydantoin) and incubated, shaking at 25°C for 24 hours, after which serial dilutions were plated onto HMM agar and incubated for 5 days at 25°C. Resulting colonies, which utilised hydantoins as a sole nitrogen source, were purified by re-streaking onto HMM agar. Isolated strains were examined for the presence of hydantoinase and *N*-carbamylamino acid amidohydrolase activity using resting cell biocatalytic assays. The wild-type *Agrobacterium* sp strain RU-OR, which was among these isolates, was identified through determination of its 16S rRNA gene sequence (shown in Figure 1) as described in Hartley *et al.* (1998).

Mutant RU-ORPN1 was selected as follows: *Agrobacterium* RU-OR cells were cultured in HMM broth to mid-log phase and then subjected to mutagenesis using

ethylmethane sulfonate (EMS) according to the method described in Miller (1992). Mutated cells were plated onto MM agar supplemented with 0.1% $(NH_4)_2SO_4$ and 0.1% 5-fluorouracil. Strain RU-ORPN1 was isolated from these plates and evaluated under standard culture and assay conditions for enzyme activity in the absence of inducer. Strain RU-ORPN1F9 was isolated by mutagenizing RU-ORPN1 cells as described above and after penicillin-enrichment for glutamine-dependent growth, cells were plated onto HMM agar supplemented with 0.002% glutamine. *Gln*$^-$ mutants were selected by relica plating to HMM without supplementation with glutamine.

GLUTAMINE SYNTHETASE ASSAYS.

Total glutamine synthetase activity was measured using the γ-glutamyl transferase assay. Cells were prepared by treatment with 0.01% cetyl-trimethylammonium bromide for 10 minutes before harvesting. The cells were then washed twice with 0.1M phosphate buffer pH 9.0 before being suspended in 50 times less volume of resuspension buffer, and assayed according to the method of Bender *et al.* (1977). Protein concentration was determined by the method of Bradford (1976). Activity is expressed as μmoles of γ-glutamyl hydroxamate generated per minute per milligram protein. The percentage adenylation of the glutamine synthetase enzyme subunits was measured using the method of Magasanik *et al.* (1995), which compares γ-glutamyl transferase in the presence and absence of magnesium ions. Magnesium ions inhibit the activity of adenylated enzyme subunits and the difference can then be used to calculate the percentage adenylation of the glutamine synthetase enzyme.

REGULATION OF HYDANTOINASE AND NCAAH ACTIVITY

Hydantoinase and NCAAH activities in *A. tumefaciens* RU-OR cells could be detected only in early stationary phase during batch culture in a complete growth medium (nutrient broth). Furthermore, enzyme activity was dependent upon growth in the presence of the hydantoin-analogue 2-thiouracil. The nutritional factors responsible for regulating enzyme activity were identified by establishing standard culture conditions under which enzyme activity was not limited to stationary phase. Hydantoinase and NCAAH activities were measured during growth of RU-OR cells in a chemically defined minimal medium containing hydantoin and glucose as sole

nitrogen and carbon sources, respectively (MM plus 0.1 % hydantoin). Activity of both enzymes was low in early exponential phase and after the cells reached stationary phase, with highest activity detected during mid to late exponential phase (Figure 2).

In all subsequent experiments, enzyme activities were determined in cells harvested during mid-exponential phase at OD_{600} = 0.5 - 0.8.

The effect of different carbon and nitrogen sources upon hydantoin-hydrolysing enzyme activity was determined by examining growth-rate and assaying for biocatalytic activity at mid-exponential growth phase. Cells were grown in minimal medium containing either glucose or glycerol as carbon source and hydantoin as nitrogen source. The growth-rate of RU-OR cells was not significantly affected by either carbon source (Figure 3) and there was also little difference in hydantoinase and NCAAH activity (Table 1).

Table 1. Hydantoin-hydrolysing activity in RU-OR cells grown with different carbon and nitrogen sources.

Carbon Source	Nitrogen Source	Hydantoinase Activity (µmol/ml)	NCAAH Activity (µmol/ml)
1% glucose	1 % hydantoin	4.87 ± 0.400	5.77 ± 0.55
1% glycerol	1 % hydantoin	3.97 ± 0.58	5.85 ± 0.58
1% glucose	0.1% $(NH_4)_2SO_4$	1.15 ± 0.2	1.09 ± 0.16
1% glucose	0.1% serine	4.70 ± 0.26	3.70 ± 0.56*
1% glucose	0.01% CAA	10.87 ± 0.43	8.68 ± 0.61

± - SEM (n = 3). * Measured as the amount of glycine generated from hydantoin as substrate. CAA - casamino acids.

In contrast, the growth rate of RU-OR cells appeared to be dramatically affected by the choice of nitrogen source. Hydantoin was the most growth-rate-limiting while 0.1% $(NH_4)_2SO_4$ and 0.1% serine were the least growth-rate limiting sources of nitrogen (Figure 3). Cells in medium containing 0.01% casamino acids, grew at an intermediate rate. The highest enzyme activity was detected in cells growing in

0.01% casamino acids and the lowest in $(NH_4)_2SO_4$. Cells grown with serine or hydantoin as a nitrogen source showed intermediate levels of enzyme activity (Table 1): growth of cells in medium containing $(NH_4)_2SO_4$ had a repressive effect upon hydantoinase and NCAAH activity (nitrogen repression).

Induced RU-OR cells (grown in SMM plus 0.1% thiouracil) were resuspended and grown in AMM plus 2-thiouracil (ammonia shock). Within 30 minutes, the hydantoinase activity had dropped three-fold, and a corresponding two-fold drop in NCAAH activity was observed (Figure 4).

When induced cells were resuspended and grown in AMM containing the glutamine synthetase inhibitor, D,L-methionine D,L-sulfoximine (MSX), there was very little drop in both hydantoinase and NCAAH activities (Figure 4), indicating that the loss of hydantoinase and NCAAH activity in RU-OR cells after ammonia shock is dependent upon glutamine synthetase activity. Induced cells were subjected to ammonia shock for 30 minutes, after which they were washed and resuspended in SMM plus thiouracil and grown for a further 60 minutes before assaying for enzyme activity. Hydantoinase and NCAAH activity returned to levels observed before ammonia shock suggesting that the ammonia shock effect could be reversed rapidly in the absence of $(NH_4)_2SO_4$. Together, this data indicates that hydantoinase and NCAAH activity in wild-type *Agrobacterium* RU-OR is dependent upon the presence of a) inducer and b) the nitrogen source in the growth medium.

CHARACTERIZATION OF MUTANT STRAINS.

Inducer-independent hydantoinase and *N*-carbamylamino acid amidohydrolase, activity was assessed by measuring enzyme activity in cells grown in SMM without 2-thiouracil. RU-ORPN1 cells showed a significant (three-fold) increase in hydantoinase activity and NCAAH activity was equivalent to induced levels in *Agrobacterium* RU-OR cells.

Table 2. Hydantoin-hydrolysing activity of mutant RU-OR strains

Strain	HYDANTOINASE *N*-carbamylglycine plus glycine (µmol/ml)		NCAAH Glycine (µmol/ml)	
	no inducer	2-thiouracil	no inducer	2-thiouracil
RU-OR (wt)	1.98±0.65	7.51±0.37	2.62±0.15	11.74±0.80
RU-ORPN1	21.8±0.78	nd	8.04±0.35	nd

± - SEM (n = 3). nd – not determined.

RU-ORPN1 cells grown in minimal medium with $(NH_4)_2SO_4$ as nitrogen source had repressed levels of hydantoinase activity, as observed in the wild-type, RU-OR cells (Figure 5), but, in contrast to the RU-OR, NCAAH activity in RU-ORPN1 cells was elevated to wild-type, induced levels (Figure 6). After growth in SMM for 60 minutes, hydantoinase activity in mutant RU-ORPN1 cells recovered to levels normally observed in induced wild-type cells (see table 2) while there was no increase in hydantoinase activity in the wild-type *Agrobacterium* RU-OR cells after growth in SMM. Thus, unlike the wild-type, the mutant strain expresses both hydantoinase and *N*-carbamylamino acid amidohydrolase enzymes even under nitrogen repression conditions, but the hydantoinase enzyme is inactive in the presence of $(NH_4)_2SO_4$.

Inhibition of glutamine synthesis reduces the sensitivity of hydantoinase activity to ammonia shock in RU-OR cells (Figure 4). Therefore, the *gln*$^-$ auxotrophic mutant RU-ORPN1F9 was subjected to ammonia shock and hydantoinase activity in the auxotrophic mutant. Figure 7 shows that hydantoinase activity in mutant RU-ORPN1F9 is no longer sensitive to ammonia shock as compared to that of the wild-type *Agrobacterium* RU-OR and mutant RU-ORPN1.

Glutamine synthetase assays of all three strains before and after ammonia shock showed that glutamine synthesis was reduced by 60% in RU-ORPN1F9 when compared to that in *Agrobacterium* RU-OR and RU-ORPN1 cells. Thus a reduction in glutamine synthesis when RU-ORPN1F9 cells are grown in $(NH_4)_2SO_4$, results in insensitivity of hydantoinase activity to ammonia shock.

HYDANTOINASE AND NCAAH ACTIVITY IN REGULATORY MUTANTS DURING GROWTH IN $(NH_4)_2SO_4$.

The hydantoinase and NCAAH activity of RU-ORPN1 and RU-ORPN1F9 cells were assessed during batch culture in SMM and compared with enzyme activity of the wild-type *Agrobacterium* RU-OR grown in the same medium, supplemented with 2-thiouracil.

Hydantoinase activity in mutant strain RU-ORPN1 followed the same trend as in the wild-type *Agrobacterium* RU-OR (Figure 8), but high levels of activity were detected in exponential growth phase in RU-ORPN1F9 cells. NCAAH activities in strains RU-ORPN1 and RU-ORPN1F9 were highest in exponential growth phase and these levels declined during stationary phase. RU-ORPN1F9 cells achieved the highest overall hydantoin-hydrolyzing activity of all three strains during exponential growth phase (Figures 8 and 9) indicating that the *gln*$^-$ phenotype does not have a deleterious effect upon hydantoinase or NCAAH production in this strain. Strain *Agrobacterium* RU-OR was selected for its efficient conversion of D,L-*p*-hydroxyphenylhydantoin to D-*p*-hydroxyphenylglycine. High levels of D,L-*p*-hydroxyphenylhydantoin-hydrolysis were also achieved. The highest D,L-*p*-hydroxyphenylhydantoin conversion by the wild-type *Agrobacterium* RU-OR and RU-ORPN1 cells was detected during stationary growth phase. In strain RU-ORPN1F9 both hydantoinase and NCAAH activity during exponential growth phase exceeded that detected in either *Agrobacterium* RU-OR or RU-ORPN1 cells Up to 45 % of D,L-*p*-hydroxyphenylhydantoin was converted to either *N*-carbamyl-*p*-hydroxyphenylglycine or D-*p*-hydroxyphenylglycine by RU-ORPN1F9 cells within six hours. RU-ORPN1F9 cells produced approximately 6 µmoles/ml D-*p*-hydroxyphenylglycine after six hours, which corresponds to 25 % conversion of D,L-*p*-hydroxyphenylhydantoin.

Figure 10 (A – C) depicts the specific hydantoinase activity per milligram dry cell mass with D,L-*p*-hydroxyphenylhydantoin as substrate. Strain RU-ORPN1 shows an overall increase of 50% in hydantoinase activity compared with wild-type *Agrobacterium* RU-OR. Mutant RU-ORPN1F9 showed the highest specific

hydantoinase activity with a 300% and 200% increase over the wild-type *Agrobacterium* RU-OR and mutant RU-ORPN1 respectively. Most important, the highest specific hydantoinase activity per unit biomass was observed in RU-ORPN1F9 cells during mid-logarithmic growth phase (0.015 units) versus 0.002 units and 0.003 units of activity in RU-OR and RU-ORPN1 cells, respectively, during the same growth phase.

CLAIMS

1. A biologically pure culture of a mutant strain of micro-organism which constitutively expresses a stereoselective enzyme system for use in the enzymatic synthesis of D-amino acids.
2. A biologically pure culture glutamine deficient micro-organism able constitutively to produce enzymes which convert racemic mixtures of 5-substituted hydantoins to D-amino acids.
3. A micro-organism able constitutively to produce enzymes which convert racemic mixtures of N-carbamylamino acids to D-amino acids.
4. A micro-organism able constitutively to produce enzymes which convert racemic mixtures of N-carbamylamino acids to D-amino acids.
5. A micro-organism as claimed in any one of claims 1 to 3 wherein the micro-organism is *Agrobacterium* sp.
6. A micro-organism as claimed in any one of claims 1 to 4 wherein the micro-organism is indistinguishable from *Agrobacterium* RU-OR based on its 16S rRNA gene sequence.
7. An isolated and purified enzyme system able to convert racemic mixtures of 5-substituted hydantoins to D-amino acids where the enzyme system is isolated and purified from a micro-organism as claimed in any one of claims 1 to 3.
8. An isolated and purified enzyme system able to convert racemic mixtures of *N*-carbamylamino acids to D-amino acids where the enzyme system is isolated and purified from a micro-organism as claimed in any one of claims 1 to 3.
9. A micro-organism as claimed in any one of claims 1 to 3 for use in the production of D-amino acids for use in the production of pharmaceuticals.
10. A micro-organism as claimed in any one of claims 1 to 3 for use in the production of D-amino acids for use in the production of agrochemicals.
11. A micro-organism as claimed in any one of claims 1 to 3 for use in the production of D-amino acids for use in the production of pesticides.
12. A micro-organism as claimed in any one of claims 1 to 3 for use in the production of D-amino acids for use in the production of feedstock additives.

13. A growth medium for use in the production of a micro-organism constitutively producing an enzyme system catalysing the conversion of 5-substituted hydantoins to D-amino acids by a micro-organism as claimed in any one of claims 1 to 3.

14. A growth medium for use in the production of a micro-organism constitutively producing an enzyme system catalysing the conversion of *N*-carbamylamino acids to D-amino acids by a micro-organism as claimed in any one of claims 1 to 3.

15. A growth medium for use in the production of micro-organisms as claimed in any one of claims 1 to 4 producing an enzyme system as claimed in either one of claims 5 or 6.

16. A growth medium as claimed in any one of claims 1 to 13 for the production of D-Amino acids from 5-substituted hydantoins during fermentation conditions.

17. A growth medium as claimed in any one of claims 1 to 13 for the production of D-Amino acids from *N*-carbamoylamino acids during fermentation conditions.

18. A growth medium for use under fermentation conditions to achieve over-expressed levels of enzyme activity for the conversion of racemic mixtures of 5-substituted hydantoins to D-amino acids by a micro-organism as claimed in any one of claims 1 to 3.

19. A growth medium for use under fermentation conditions to achieve over-expressed levels of enzyme activity for the conversion of racemic mixtures of *N*-carbamylamino acids to D-amino acids by a micro-organism as claimed in any one of claims 1 to 3.

20. A *N*-carbamylamino acid produced in accordance with the invention.

21. A D-amino acid produced in accordance with the invention.

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Figure 1

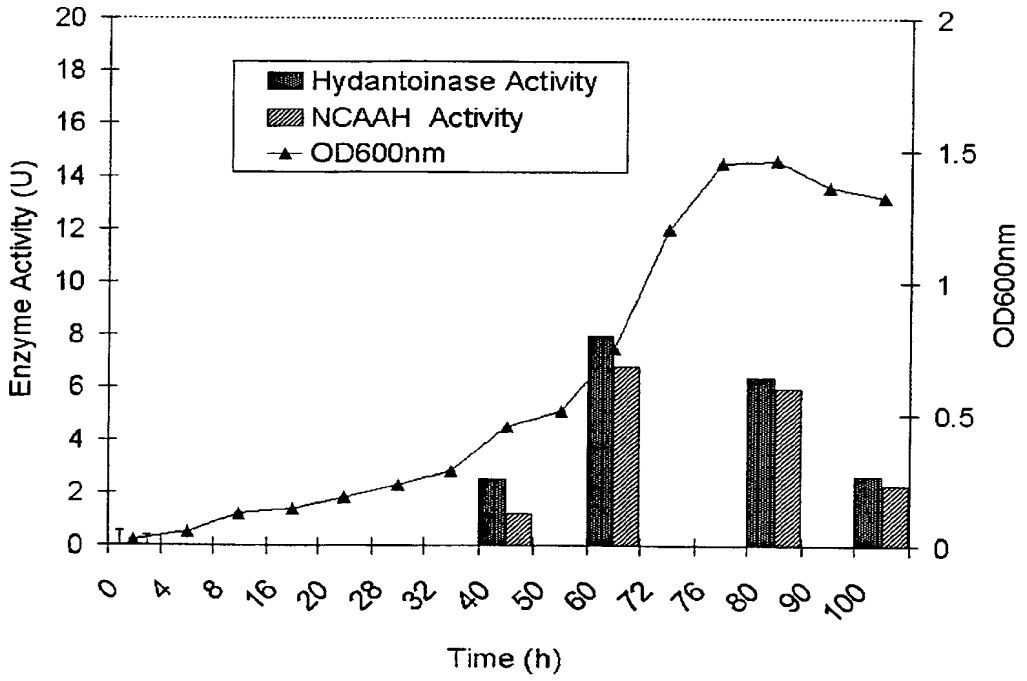


Figure 2

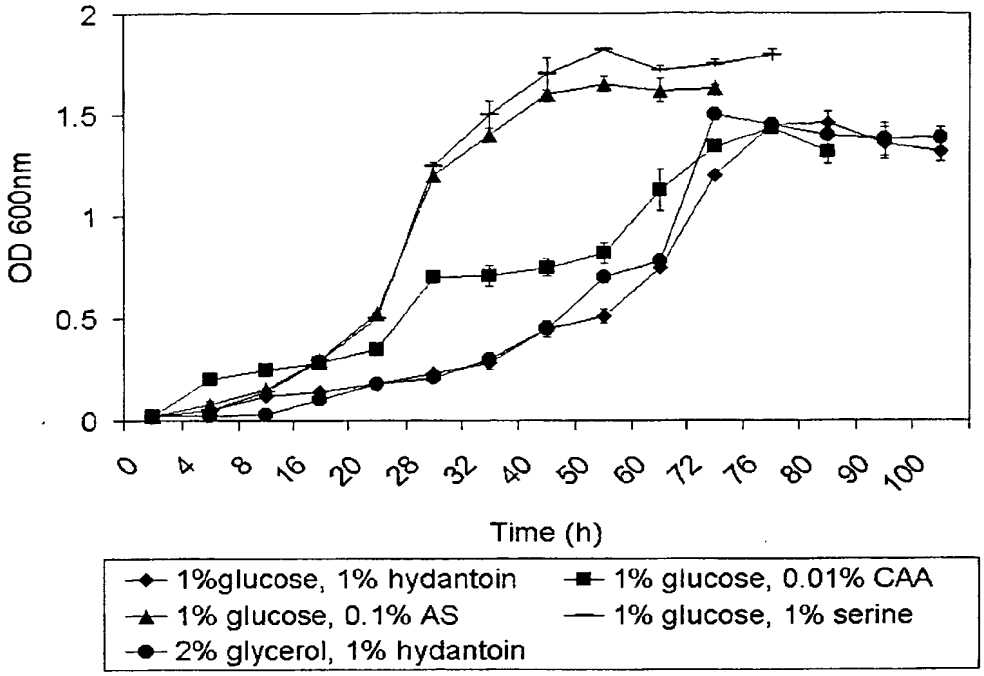


Figure 3

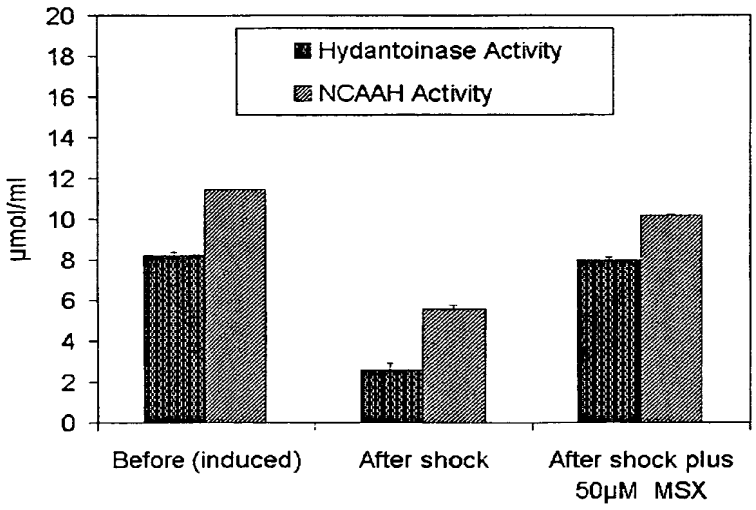


Figure 4

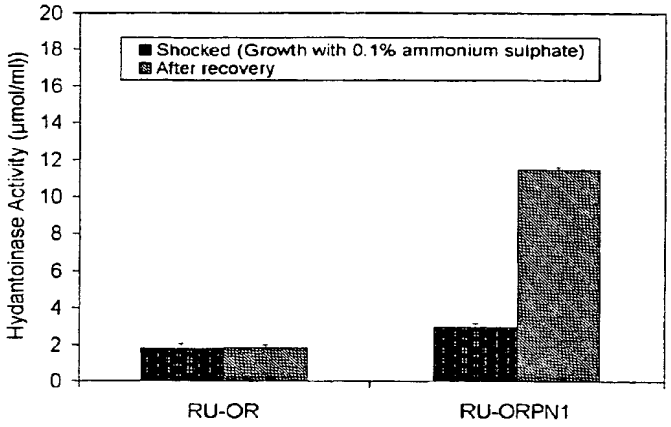


Figure 5

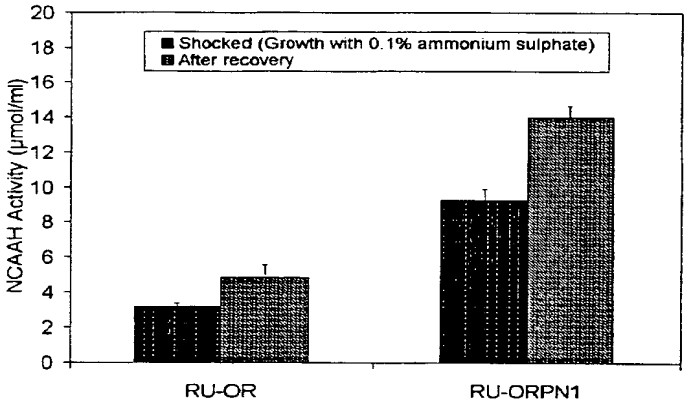


Figure 6

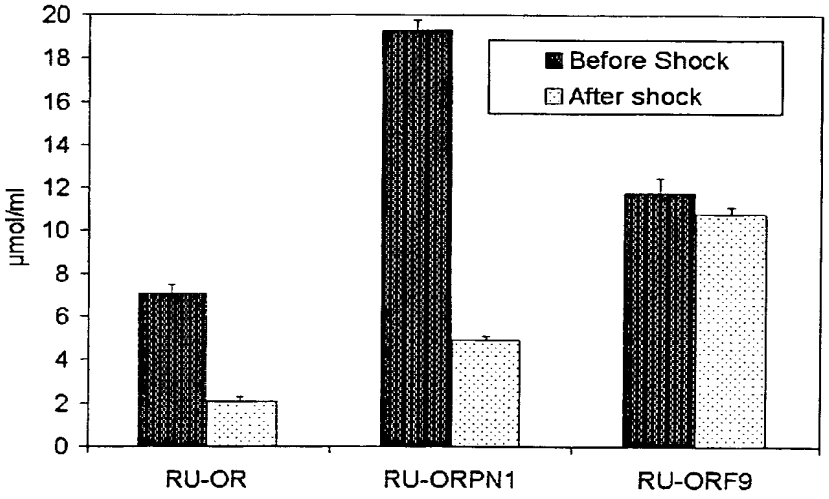


Figure 7

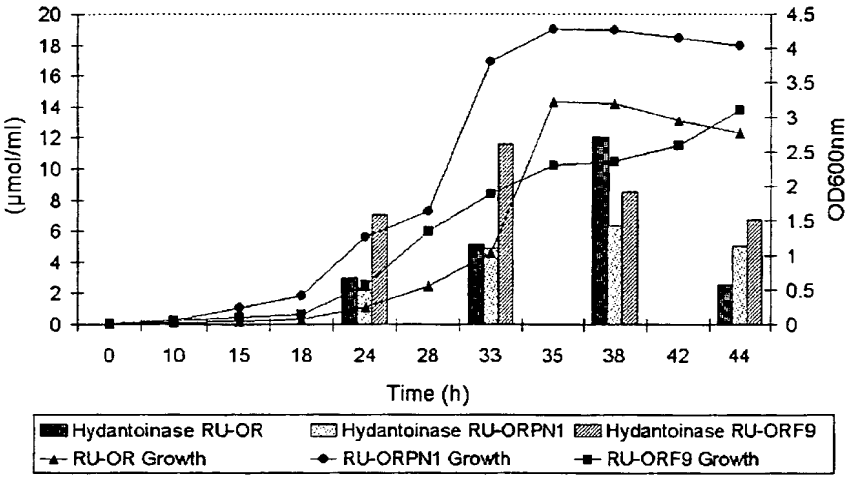


Figure 8

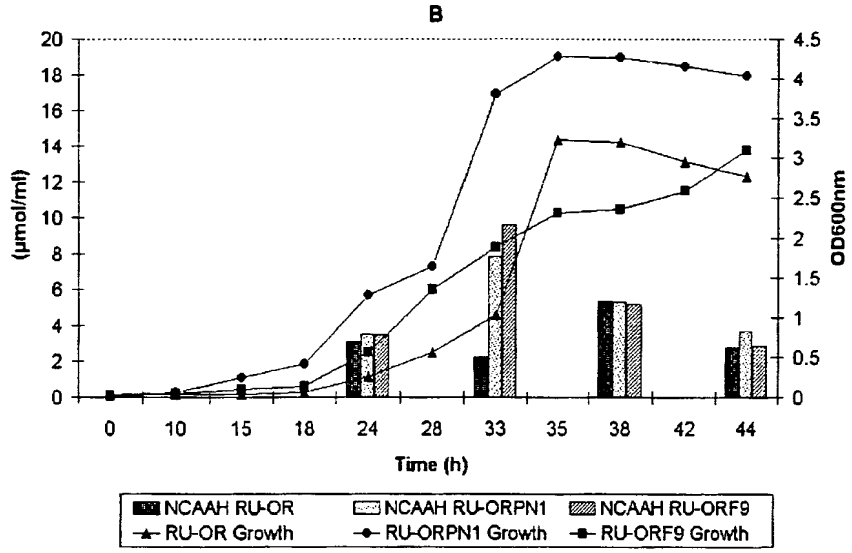
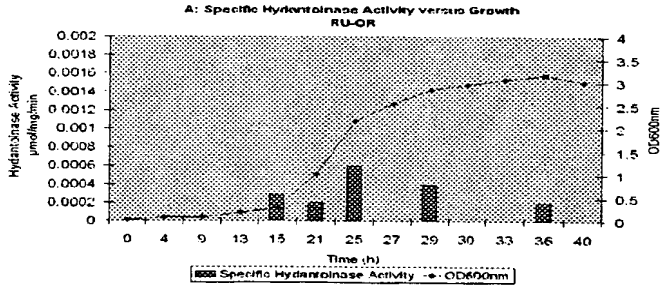
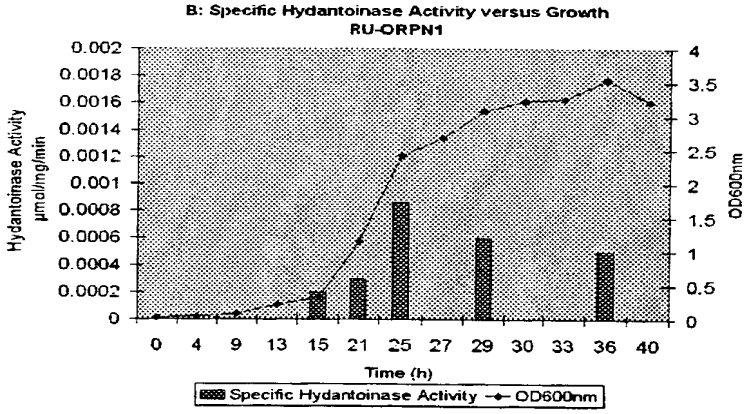


Figure 9


A: Specific Hydantoinase Activity versus Growth
RU-OR
Hydantoinase Activity µmol/mg/min
OD600nm
Time (h)
Specific Hydantoinase Activity
OD600nm


B: Specific Hydantoinase Activity versus Growth
RU-ORPN1
Hydantoinase Activity µmol/mg/min
OD600nm
Time (h)
Specific Hydantoinase Activity
OD600nm

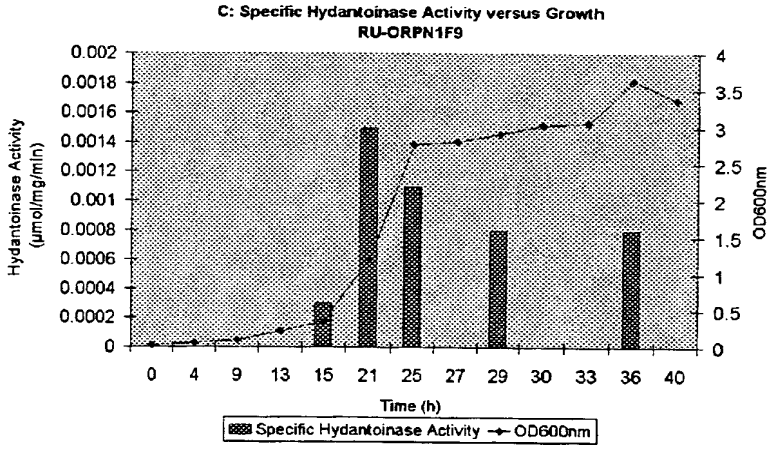

C: Specific Hydantoinase Activity versus Growth
RU-ORPN1F9
Hydantoinase Activity (µmol/mg/min)
OD600nm
Time (h)
Specific Hydantoinase Activity
OD600nm

Figure 10

PATENT COOPERATION TREATY

PCT 12 SEP 2001

PCT

14

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P14078PC00		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/ZA00/00173	18/09/2000	17/09/1999
International Patent Classification (IPC) or national classification and IPC		
C12N15/11		
Applicant		
BURTON, Stephanie, Gail; et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12/04/2001	10.09.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Dumont, E Telephone No. +49 89 2399 7704

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/ZA00/00173

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-13	as originally filed				

Claims, No.:

1-11	as received on	24/08/2001	with letter of	15/08/2001	

Drawings, sheets:

1/6-6/6	as originally filed				

Sequence listing part of the description, pages:

1, Seq. 1, filed with letter of 19.01.2001, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☒ furnished subsequently to this Authority in written form.
   - ☒ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

☐ the description, pages:

☒ the claims, Nos.: 1-21 as originally filed

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 2
	No:	Claims 1, 3-11
Inventive step (IS)	Yes:	Claims 2
	No:	Claims 1, 3-11
Industrial applicability (IA)	Yes:	Claims 1-11
	No:	Claims

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents cited in the International Search Report (ISR):

D1:HARTLEY C J ET AL.

D2:EP-A-0 677 585

1. The present application discloses an alternative solution for the microbial production of optically pure D-amino acids. The main problem when using homologous hosts for the expression of hydantoinase and N-carbamylamino acid amidohydrolase (NCAAH), the enzymes involved in the bioconversion of hydantoins to enantiomerically pure amino acids, is that the enzyme activity needs to be induced. The invention concerns two mutants of the wild-type *Agrobacterium* sp. strain RU-OR, named RU-ORPN1 and RU-ORPN1F9, which show enzyme activity in the absence of inducer. Hydantoinase activity of the RU-ORPN1F9 mutant is, in addition, insensitive to ammonia shock. This is shown to correlate with a reduction of glutamine synthesis in this mutant.

2. Novelty (Art. 33(2) PCT)

Claims 1, 3-11 lack novelty in view of D1 and D2 for the following reasons: D1 discloses a mutant of wild-type *Agrobacterium* sp. strain RU-OR, RU-ORL5, which shows constitutive expression of both hydantoinase and NCAAH (p. 708, column 1, lines 4-9). This mutant can convert racemic mixtures of 5-substituted hydantoins or N-carbamylamino acids to their respective D-amino acids, and is suitable for use in the industrial production of D-amino acids (p. 711, column 1, last paragraph). RU-ORL5 is presumably indistinguishable from *Agrobacterium* RU-OR based on its 16S rRNA gene sequence. Thus, claims 1, 3-5 and 10-11 lack novelty.

It is pointed out that the word "constitutive" is hereby understood to mean an "inducer-independant" mutant, since the enzyme activity of mutant strain RU-ORPN1 disclosed in the present application is characterized as being "constitutive", although it is repressed by ammonium in the growth medium (description p. 5, lines 29-31).

D2 teaches the production of microorganisms transformed with a plasmid capable of expressing at high levels and without inducers an enzymatic system consisting of D-hydantoinase and D-NCAAH, and the use of this enzymatic system isolated from said microorganisms for the production of D-amino acids. Although the present application does not concern genetically modified organisms but mutant strains, enzyme systems

isolated from both types of organisms would be indistinguishable. Therefore D2 is novelty-destroying for claims 6 and 7 (see also "additional remarks to item VIII", 1.).
A growth medium suitable for the production of RU-ORL5 strain is known from D1. A medium for use under fermentation conditions to achieve high yields of conversion of 5-substituted hydantoins or N-carbamylamino acids to D-amino acids in microorganisms constitutively expressing the enzyme system is known from D2. Therefore claims 8 and 9 lack novelty (see also "additional remarks to item VIII", 2.).

3. Inventive step (Art.33(3) PCT)
With regard to the result of the ISR, the subject-matter of claim 2 appears to be novel and to involve an inventive step, since a correlation between the drop in activity of both hydantoinase and NCAAH and the glutamine synthetase activity has not been disclosed in the cited prior art. The intentional isolation of a mutant with reduced glutamine synthesis, this reduction correlating with a highly efficient and nitrogen insensitive activity of hydantoinase and NCAAH, is considered to involve an inventive step.

Additional remarks to item VII
A reference list of the documents cited in the description is missing in the application.

Additional remarks to item VIII
Clarity of the claims (Art. 6 PCT)
1. The products in claims 6-7 and 10-11 are defined in terms of a process. The attention of the applicant is drawn to the fact that no unified criteria exist in PCT for the assessment of a "product-by-process" claim. The EPO, for example, considers that a process feature in a product claim can only be relied on for establishing novelty over the prior art, where the use of that process necessarily means that the product has a particular characteristic and the skilled person following the teaching of the application would inevitably achieve that characteristic, would be aware of that characteristic, and would discard any product not having it.
2. Regarding claims 8 and 9, the applicant is reminded that in general a claim to a substance for a particular use is construed as meaning a substance or composition which is in fact suitable for the stated use (PCT International Preliminary Examination Guidelines, as in force from 09.10.98, Section IV, paragraph III-4.8).

CLAIMS

1. A biologically pure culture of a mutant strain of an *Agrobacterium* sp. which constitutively expresses a stereoselective enzyme system for use in the enzymatic synthesis of D-amino acids.
2. The *Agrobacterium* sp. as claimed in claim 1 which is glutamine-deficiently able constitutively to produce enzymes which convert racemic mixtures of 5-substituted hydantoins to D-amino acids.
3. The *Agrobacterium* sp. as claimed in either claim 1 or 2 able constitutively to produce enzymes which convert racemic mixtures of *N*-carbamylamino acids to D-amino acids.
4. The *Agrobacterium* sp. as claimed in any one of claims 1 to 3 which is indistinguishable from *Agrobacterium* RU-OR based on its 16S rRNA gene sequence.
5. Use of the *Agrobacterium* sp. as claimed in any one of claims 1 to 4 in the production of a chemical selected from the group consisting of pharmaceuticals, agrochemicals, pesticides and feedstock additives.
6. An isolated and purified enzyme system able to convert racemic mixtures of 5-substituted hydantoins to D-amino acids where the enzyme system is isolated and purified from the *Agrobacterium* sp. as claimed in any one of claims 1 to 4.
7. An isolated and purified enzyme system able to convert racemic mixtures of *N*-carbamylamino acids to D-amino acids where the enzyme system is isolated and purified from the *Agrobacterium* sp. as claimed in any one of claims 1 to 4.
8. A growth medium for use in the production of the *Agrobacterium* sp. as claimed in any one of claims 1 to 4.
9. The growth medium as claimed in claim 8 for use under fermentation conditions to achieve over-expressed levels of the enzyme system as claimed in either claim 6 or 7.
10. A *N*-carbamylamino acid produced in accordance with the invention.
11. A D-amino acid produced in accordance with the invention.

AMENDED SHEET

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
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(51) International Patent Classification[7]: C12N 15/11, 1/20, C12P 13/04, 41/00, C12R 1/01, C12Q 1/68 // (C12P 13/04, C12R 1:01) (C12P 41/00, C12R 1:01)

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18 September 2000 (18.09.2000)

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(71) Applicant *(for all designated States except US)*: **AECI LIMITED** [ZA/ZA]; 24 The Woodlands, Woodlands Drive, Woodmeads, 2128 Johannesburg (ZA).

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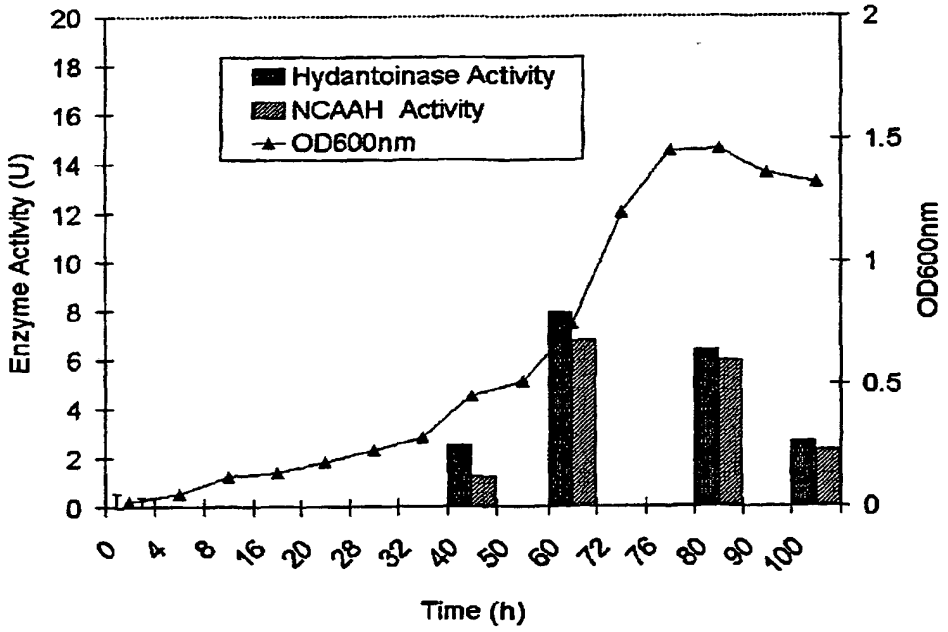
(74) Agent: **JOHN & KERNICK**; An Intellectual Property Office of Bowman Gilfillan Inc., P.O. Box 3511, 1685 Halfway House (ZA).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

[Continued on next page]

(54) Title: MICRO-ORGANISMS, THEIR USE AND METHOD FOR PRODUCING D-AMINO ACIDS



(57) Abstract: The invention relates to novel micro-organisms which are simple to cultivate and their use in the production of D-amino acids, particularly micro-organisms suitable for the production of D-amino acids from corresponding hyantoins of *N*-carbamoylamino acids.

WO 01/19982 A3

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
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(88) **Date of publication of the international search report:**
25 October 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/ZA 00/00173

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 C12N1/20 C12P13/04 C12P41/00 C12R1/01
C12Q1/68 //(C12P13/04,C12R1:01),(C12P41/00,C12R1:01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P C12R C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBL, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HARTLEY C J ET AL.: "Production of D-amino acids from D,L-5-substituted hydantoins by an Agrobacterium tumefaciens strains and isolation of a mutant with inducer-independent expression of hydantoin-hydrolising activity" BIOTECHNOLOGY LETTERS, vol. 20, no. 7, July 1998 (1998-07), pages 707-711, XP000944140 cited in the application abstract page 707, right-hand column, line 10 -page 708, left-hand column, line 9 page 710, left-hand column, line 1 -right-hand column, line 7; table 3 page 711, left-hand column, line 19 -right-hand column, line 3 --- -/--	1,3-21

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 April 2001	16/05/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van de Kamp, M

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 677 585 A (ENIRICERCHE SPA) 18 October 1995 (1995-10-18) page 3, line 1-4 page 3, line 48 -page 4, line 14 examples 10-14	1,3,4, 7-21
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A	DEEPA S ET AL.: "Enzymatic production and isolation of D-amino acids from the corresponding 5-substituted hydantoins;" PROCESS BIOCHEMISTRY, vol. 28, no. 7, 1993, pages 447-452, XP000997712 cited in the application figure 5; table 1	13-19
A	ACHARY A ET AL.: "Application of numerical modeling for the development of optimized complex medium for D-hydantoinase production from Agrobacterium radiobacter NRRL B 11291;" BIOTECHNOL. BIOENG., vol. 55, no. 1, 1997, pages 148-154, XP002165711 the whole document	13-19
A	BURTON S G ET AL.: "Production of enantiomerically pure amino acids: Characterisation of South African hydantoinases and hydantoinase -producing bacteria." JOURNAL OF MOLECULAR CATALYSIS B ENZYMATIC, vol. 5, no. 1-4, 15 September 1998 (1998-09-15), pages 301-305, XP000997486 abstract page 304, right-hand column, line 3 -page 305, left-hand column, line 4	13-19

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INTERNATIONAL SEARCH REPORT

Inte[redacted]l Application No
PCT/ZA 00/00173

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0677585 A	18-10-1995	IT 1274167 B	15-07-1997
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		DE 69500450 T	22-01-1998
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		ES 2106588 T	01-11-1997
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		ES 2052760 T	16-07-1994

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
P14078PC00		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/ZA 00/ 00173	18/09/2000	17/09/1999
Applicant		
BURTON, Stephanie, Gail; et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   MICRO-ORGANISMS, THEIR USE AND METHOD FOR PRODUCING D-AMINO ACIDS

5. With regard to the **abstract,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 2

   [ ] as suggested by the applicant.

   [X] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

B x III TEXT OF THE ABSTRACT (Continuation of item 5 of th first sheet)

The invention relates to micro-organisms which are simple to cultivate and their use in the production of D-amino acids, particularly micro-organisms suitable for the production of D-amino acids from corresponding hyantoins of N-carbamoylamino acids.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/ZA 00/00173

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 C12N1/20 C12P13/04 C12P41/00 C12R1/01
C12Q1/68 //(C12P13/04,C12R1:01),(C12P41/00,C12R1:01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P C12R C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBL, MEDLINE, EMBASE

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 April 2001	16/05/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van de Kamp, M

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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3

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3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/ZA 00/00173

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0677585 A	18-10-1995	IT 1274167 B	15-07-1997
		AT 155814 T	15-08-1997
		DE 69500450 D	28-08-1997
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		AT 83504 T	15-01-1993
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